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**Neuromuscular development in *Novocrania anomala*: evidence for the presence of serotonin and a spiralian-like apical organ in lecithotrophic brachiopod larvae**

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## ABSTRACT

The phylogenetic position of Brachiopoda remains unsettled, and only few recent data on brachiopod organogenesis are currently available. In order to contribute to questions concerning brachiopod ontogeny and evolution we investigated nervous and muscle system development in the craniiform (inarticulate) brachiopod *Novocrania anomala*.

Larvae of this species are lecithotrophic and have a bilobed body with three pairs of dorsal setal bundles which emerge from the posterior lobe. Fully developed larvae exhibit a network of setae pouch muscles as well as medioventral longitudinal and transversal muscles. After settlement, the anterior and posterior adductor muscles and delicate mantle retractor muscles start to form. Comparison of the larval muscular system of *Novocrania* with that of rhynchonelliform (articulate) brachiopod larvae shows that the former has a much simpler muscular organization.

The first signal of the neurotransmitter serotonin appears in fully developed *Novocrania* larvae, which have an apical organ that consists of four flask-shaped cells and two ventral neurites. These ventral neurites do not stain positively for the axonal marker  $\alpha$ -tubulin in the larval stages. In the juveniles, the nervous system stained by  $\alpha$ -tubulin is characterized by two ventral neurite bundles with a commissure in the mid-body region. Our data are the first direct proof for the presence of an immunoreactive neurotransmitter in lecithotrophic brachiopod larvae and demonstrate the existence of flask-shaped serotonergic cells in the brachiopod larval apical organ, thus significantly increasing the probability that this cell type was part of the bauplan of the larvae of the last common lophotrochozoan ancestor.

Key words: Lophotrochozoa, evolution, myogenesis, neurogenesis, Bilateria

## INTRODUCTION

The phylogenetic position of Brachiopoda remains unresolved, although most molecular analyses agree on their inclusion within Lophotrochozoa. Thereby, some recent works support the more traditional view that Brachiopoda clusters with Ectoprocta and Phoronida to form the Lophophorata, the direct sistergroup of Spiralia (Trochozoa) (Gee 1995; Nielsen 2002; Halanych 2004). Current brachiopod internal phylogeny suggests division of the phylum into the three clades Linguliformea, Craniiformea, and Rhynchonelliformea (Williams et al. 1996). Craniiform brachiopods share morphological traits with both linguliforms and rhynchonelliforms. As such, a circumferential mantle cavity, a muscle system with oblique muscles and two pairs of shell adductors, a transitional median tentacle during lophophore development, and a median division of the brachial canals into two separate cavities within the lophophore may be regarded as common features of craniiforms and linguliforms, while a proteinaceous calcitic shell, a single row of tentacles on a trocholophous lophophore, gonads suspended in the mantle sinus, and lecithotrophic larvae are shared between craniiforms and rhynchonelliforms (Rowell 1960; Atkins and Rudwick 1962; Williams et al. 1996).

Experimental embryology has shown that the animal half of the egg forms the ectodermal epithelium of the apical lobe, while the vegetal half forms endoderm, mesoderm and the ectoderm of the mantle lobe in *N. anomala* (previously assigned to various genera and thus referred to in the literature as *Crania anomala* or *Neocrania anomala*, respectively) (Lee and Brunton 1986, 2001; Freeman 2000). During metamorphosis, both the ventral and the dorsal shell are formed from the dorsal epithelium of the larva (Nielsen 1991).

Recent immunocytochemical studies have revealed the almost universal occurrence of an apical organ that contains flask-shaped cells in larvae of Annelida, Mollusca, Sipuncula, Entoprocta, and Platyhelminthes (see Wanninger 2009 for review). These flask-shaped cells usually contain the neurotransmitter serotonin and may also show FMRFamide-like immunoreactivity. The wide occurrence of serotonin throughout the Metazoa indicates that this transmitter was part of the ancestral nervous system of multicellular animals (Hay-Schmidt 2000). Surprisingly, neither the serotonin molecule itself nor the existence of flask-shaped cells has hitherto been proven for lecithotrophic larvae of any brachiopod clade, thus leaving a significant gap in our understanding of the evolution of the brachiopod nervous system and the origin of this cell type within the lophophorates. Accordingly, we provide herein the first thorough immunocytochemical study on neurogenesis in a brachiopod with a lecithotrophic larva, the craniiform *Novocrania anomala*, and compare our findings with data on other lophotrochozoan phyla such as mollusks and annelids. In our general quest to shed light on brachiopod organogenesis, we also present data on *Novocrania* myogenesis, which for the first time allows conclusive comparisons between the muscular systems of craniiform and rhynchonelliform brachiopod larvae and thus contributes to questions concerning the ancestral muscular bodyplan of brachiopod larvae.

## **MATERIAL AND METHODS**

### **Animal collection, breeding, and fixation**

Rocks with attached adults of *Novocrania anomala* were obtained by dredging in the vicinity of the Sven Lovén Centre for Marine Sciences, Gullmarsfjord, Sweden (58°15'921"N, 11°25'103"E) in October 2007 and September 2008. The rocks were maintained in the laboratory in running seawater and adults were removed and dissected for gametes. For artificial fertilization, eggs and sperm were removed from the gonads with pulled glass pipettes and separately left in beaker glasses with filtered seawater at ambient seawater temperature (14°C). The water containing the eggs was changed at least four times to wash off follicle cells and superfluous gonad tissue. Eggs were regularly checked for germinal vesicle breakdown and sperm cells were checked for motility under a compound microscope. After approximately 12 hours, 2 ml of a highly diluted sperm suspension (testes of three to five adults in approximately 100 ml filtered sea water) were added to the beaker glasses containing eggs. Developing larvae were fixed at various stages after fertilization (from 34 hours post fertilization [hpf] to 17 days post settlement) in 4% paraformaldehyde in 0.1M phosphate buffer (PB) for 90 min. Thereafter, larvae were washed three times for 15 min each in 0.1M PB and finally stored in 0.1M PB containing 0.1% NaN<sub>3</sub> at 4°C.

### **Immunocytochemistry, confocal laserscanning microscopy (CLSM), and 3D reconstruction**

Prior to staining, larvae were washed thrice for 15 min each in PB and incubated for 1 h in PB containing 0.2% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) at room temperature to permeabilize the tissue. For F-actin staining, specimens were left overnight at 4°C in 0.1M PB containing 0.2% Triton X-100 and 1:40 diluted Alexa Fluor

488 phalloidin (Invitrogen, Molecular Probes, Eugene, OR, USA). For serotonin and  $\alpha$ -tubulin staining, specimens were first incubated overnight at 4°C in 6% normal goat serum in 0.1M PB and 0.2% Triton X-100 (blocking solution). Second, specimens were incubated for 24 hours at 4°C in blocking solution containing either a 1:800 diluted polyclonal primary serotonin antibody (Zymed, Carlton Court, CA, USA), or a 1:500 diluted monoclonal primary acetylated  $\alpha$ -tubulin antibody (Sigma-Aldrich). Third, specimens were washed in the permeabilization solution overnight at 4°C with four changes. Then, the secondary antibodies (either Alexa Flour 633-conjugated goat anti-rabbit, Invitrogen, or TRITC-conjugated goat anti-rabbit, Sigma-Aldrich) were added in a 1:300 dilution to the blocking solution and the samples were incubated for 24 hours. Subsequently, the specimens were washed three times for 15 min each in 0.1M PB and embedded in Fluoromount G (Southern Biotech, Birmingham, AL, USA) on glass slides. Negative controls omitting either the phalloidin dye or the respective secondary antibody were performed in order to test for signal specificity and rendered no signal. The samples were analyzed with a Leica DM RXE 6 TL fluorescence microscope equipped with a TCS SP2 AOBS laserscanning device (Leica Microsystems, Wetzlar, Germany). Animals were scanned with 0.16  $\mu$ m - 0.49  $\mu$ m step size, and the resulting image stacks were merged into maximum projection images. In addition, light micrographs were recorded to allow overlay with the CLSM images for exact orientation and localization of the muscle and nervous systems within the animals. Adobe Photoshop CS3 software (Adobe, San Jose, CA, USA) was used to create overlay images and for assembling the figure plates. The sketch drawings were generated with Adobe Illustrator CS3 (Adobe), and the 3D



reconstructions were created with the Imaris imaging software Version 5.7.2 (Bitplane, Zürich, Switzerland) based on the CLSM image stacks.

## **RESULTS**

### **Myogenesis**

The first signals of F-actin were found in the setae pouches of bilobed larvae at the onset of setae formation. The six setae pouches are distributed in pairs along the dorsal ridge of the posterior lobe (Fig. 1A). As the setae grow, the setae pouch muscles develop further into spherical systems (Fig. 1, B and G-I; Fig. 2A). Later in development, the setae pouch muscles get interconnected by two bundles of medioventral longitudinal muscles, which run ventrally from anterior to posterior (Fig. 1, C-D and G-I; Fig. 2A). The medioventral longitudinal muscle strands get interconnected by transversal muscles (Fig. 1, B-D and G-I) which are distributed homogenously in early stages (Fig. 1B) and concentrate into three bundles in later stages (Fig. 1D). Accordingly, the metamorphic competent larva has setae pouch muscles, medioventral longitudinal muscles, and transversal muscles. During metamorphosis, the larval musculature is replaced by the juvenile musculature which most likely develops entirely *de novo*, i.e., independent of the larval muscle systems (Fig. 1E). The juvenile musculature comprises mantle margin muscles, oblique muscles, as well as anterior and posterior adductor muscles (Fig. 1F).

### **Neurogenesis**

The first signals of the neurotransmitter serotonin appear in fully developed, metamorphic competent, bilobed larvae. At this stage, four flask-shaped cells are present in the anterior-most part of the apical lobe (Fig. 3, A-D). They are oriented in different directions with only one pointing towards the apical pole of the larva. The flask-shaped cells are connected to two ventral neurites which extend posteriorly (Fig. 3, A-D). The flask-shaped cells are lost during metamorphosis, and early juveniles have two ventral neurites which project from the anterior lobe into the posterior lobe (Fig. 3E). During subsequent development, the ventral neurites get interconnected by a commissure in the mid-part of the juvenile, at the former border between the apical and the posterior lobe (Fig. 3F).

The axonal marker  $\alpha$ -tubulin is first expressed in juveniles five days after metamorphosis (Fig. 4A). Two solid neurite bundles develop ventrolaterally in the anterior lobe of the juvenile and subsequently grow in posterior direction into the posterior lobe (Fig. 4B). Later in development, these neurite bundles close anteriorly and posteriorly, and the commissure in the region of the former border between the anterior and posterior lobe is established (Fig. 4, C-F). Serially arranged mantle neurites extend from the anterior part of the ventral neurite bundles in lateral direction towards the mantle margin of the juvenile (Fig. 4, B-F). Comparison of the position of the  $\alpha$ -tubulin signal in the juveniles and the serotonin signal in the larva suggests that the larval ventral neurites form the precursors of the juvenile ventral neurite bundles.

## **DISCUSSION**

### **Comparative brachiopod myoanatomy**

The musculature of fully developed *Novocrania* larvae consists of setae pouch muscles, the medioventral longitudinal muscles that interconnect these setae pouch muscles, and transversal muscles that interconnect the medioventral longitudinal muscles. This relatively simple muscular organization differs significantly from that of articulate brachiopod larvae, which comprises pedicle muscles, longitudinal muscles, a circular mantle muscle, central mantle muscles, a U-shaped muscle, serially arranged mantle muscles, setae muscles, setae pouch muscles, an apical longitudinal, and an apical transversal muscle (Fig. 2B herein and Altenburger and Wanninger 2009). These differences may be due to different functional aspects in craniiform (inarticulate) and rhynchonelliform (articulate) larvae, especially during metamorphosis. Thereby, larvae of *Novocrania* curl ventrally by contraction of the paired medioventral muscles and attach to the substrate via the epithelium at the posterior end of the larva. The brachial valve is then secreted by the median part of the dorsal epithelium and the pedicle valve is secreted by the attachment epithelium (Nielsen 1991). Larvae of the rhynchonelliform brachiopod *Terebratalia* attach via a secretory product produced by the distal tip of the pedicle lobe at the posterior end of the larva. After attachment, the mantle lobe flips over the apical lobe and secretes a protegulum containing calcium carbonate (Stricker and Reed 1985; Freeman 1993). Despite these differences at metamorphosis, we regard it unlikely that the larval ecology of *Novocrania* and *Terebratalia* differs significantly, and we therefore argue that the distinct differences in the larval myoanatomy do not reflect functional

constraints but hint towards an early evolutionary divergence of craniiform and rhynchonelliform brachiopods.

The phylogenetic relationship of craniiforms to the other brachiopod subtaxa is still controversial. Based on their lack of a valve-to-valve articulation they have traditionally been grouped together with other inarticulated groups (Williams and Rowell 1965). This view is supported by molecular analyses based on 18S rDNA sequences, which either place the craniiforms within the linguliforms (Cohen 2000) or as the direct sistergroup of the linguliforms (Cohen and Weydmann 2005). Other morphological characters such as the presence of an anus and a lophophore without internal mineralized support underpins a close relationship of craniiform and linguliform brachiopods (Carlson 1995). However, based on the lecithotrophy of the larvae and the presence of a calcareous shell in the adults, craniiform brachiopods have been proposed to be closer related to the rhynchonelliforms rather than to the linguliforms, which have a free-swimming planktotrophic life cycle stage that closely resembles the morphology of juvenile brachiopods (Nielsen 1991). Accordingly, an alternative scenario proposes that lecithotrophic larvae equipped with larval setae are basal for Brachiopoda and that the swimming “paralarvae” of lingulids constitute a planktonic juvenile stage, thereby implying that the linguliforms have secondarily lost the lecithotrophic larva (Lüter 2001). The musculature of post- metamorphotic *Novocrania* comprises anterior adductors, posterior adductors and oblique lateral muscles. This corresponds to the musculature found in adults, which in addition have brachial protractor muscles at the base of the lophophore, an unpaired median muscle, and oblique internal muscles (Bulman 1939; Helmcke 1939; Williams and Rowell 1965). In the present study we found mantle

retractor muscles, which had previously been undescribed for *Novocrania* and which correspond to the respective muscles found in the rhynchonelliform brachiopods *Argyrotheca cordata*, *A. cistellula*, and *Terebratalia transversa* (Altenburger and Wanninger 2009).

Given the distinct differences in the larval musculature of craniiforms and rhynchonelliforms it is difficult to infer a muscular groundpattern for brachiopod larvae. However, it appears likely that a hypothetical ancestral brachiopod larva had at least setae pouch muscles and a musculature that interconnect these setae pouch muscles.

### **Neurogenesis**

The serotonergic nervous system of *Novocrania anomala* starts to develop in fully established larvae and shows an apical organ consisting of four flask-shaped cells and two lateroventral neurites, which grow from the anterior lobe into the posterior lobe. These results constitute the first unambiguous account of the presence of an apical organ with serotonergic flask-shaped cells in a lecithotrophic brachiopod larva. Similar apical organs containing flask-shaped cells have been found in a wide range of lophotrochozoan phyla including entoprocts (Wanninger et al. 2007), mollusks (Voronezhskaya et al. 2002; Wanninger and Haszprunar 2003), annelids (Voronezhskaya et al. 2003), and ectoprocts (Pires and Woollacott 1997; Shimizu et al. 2000). The finding of an apical organ with flask-shaped cells in a lecithotrophic brachiopod larva suggests that such an apical organ was also present in the larva of the last common lophotrochozoan ancestor (Wanninger 2009) and that lecithotrophic larvae are basal for Brachiopoda. Similar to the vast majority of lophotrochozoan larvae, but significantly different to the situation found

in the entoproct creeping-type larva and the larva of polyplacophoran mollusks, the apical organ of *N. anomala* is comparatively simple, thus supporting the notion that a simple apical organ was present in the “ur-lophotrochozoan” larva and that Entoprocta and Mollusca form a monophyletic assemblage (Tetraneuralia concept; see Wanninger 2009). A serotonergic nervous system has previously been described for planktotrophic linguliform brachiopod “paralarvae”. There, the apical organ is located at the base of the median tentacle and comprises numerous serotonergic cells (Hay-Schmidt 1992). While it is tempting to speculate that this neural structure might correspond to the spiralian-type apical organ described herein for *Novocrania*, it is important to note (i) that a flask-shaped character could not be assigned to the apical organ cells of these linguliform paralarvae and (ii) that the number of cells in the apical organ is considerably higher than that in the other spiralian larvae. Overall, the “apical organ” of linguliform larvae resembles more closely the one found in phoronid and deuterostome larvae (Santagata 2002), the homology of which remains to be proven. The suggested derived character of the nervous system of linguliform brachiopod paralarvae is consistent with the view that linguliforms have lost the lecithotrophic larva and have secondarily acquired a planktotrophic life cycle stage via a stage that resembles a swimming juvenile rather than a “true” brachiopod larva (Lüter 2001).

We found  $\alpha$ -tubulin-positive neural tissue solely in post-metamorphic specimens of *N. anomala*. The  $\alpha$ -tubulin signal is located in a region comparable to the serotonin signal and shows two ventral neurite bundles that are interconnected at the anterior end, at the posterior end, and by a commissure at the former border between the apical lobe and the posterior lobe. The fact that we did not find  $\alpha$ -tubulin in the *Novocrania* larvae that

exhibit serotonergic neurites demonstrates that tubulin alone is not a reliable marker for nervous structures in lophotrochozoan larvae.

The tubulinergetic nervous system in juvenile *Novocrania* outlines the adult nervous system, which consists of two ventral neurite bundles, a subesophageal and a supraesophageal commissure, and mediodorsal mantle neurites (Blochmann 1892; Bullock and Horridge 1965). The anterior ventral neurite bundles form the arm neurites of the lophophore. Perpendicular from these arm neurites extend accessory brachial neurites (Williams and Rowell 1965).

Despite some classical studies, the adult neural anatomy of brachiopods is only poorly known (James et al. 1992). In the articulate *Gryphus* the nervous system comprises a transverse supraenteric ganglion and a subenteric ganglion lying above and below the esophagus, as does the subesophageal and supraesophageal commissure in *Novocrania anomala* (Bullock and Horridge 1965). Our study provides a first step towards an understanding of the larval anatomy, neurotransmitter distribution, and development of the nervous system in brachiopod taxa with lecithotrophic larvae. While additional data are needed to assess the brachiopod neural groundpattern, the finding that serotonergic flask-shaped cells similar to those found in spiralian larvae do occur in the apical organ of *N. anomala* larvae strengthens the hypothesis that this cell type was also present in the last common ancestor of Lophotrochozoa (see Wanninger 2009).

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## FIGURE LEGENDS

**Figure 1:** Muscle development in *Novocrania anomala*. Overlay of maximum projection micrographs from phalloidin staining and light micrographs. Anterior faces upwards and scale bars equal 50  $\mu\text{m}$ . (A) Larva with anterior lobe (AL), posterior lobe (PL) and early signs of F-actin in the three pairs of setae pouches (arrows) along the dorsal ridge of the posterior lobe. (B) Larva with setae (se), anterior lobe (AL), posterior lobe (PL), setae pouch muscles (arrows), homogenously distributed transversal muscles (asterisks), and a distinct F-actin-rich area (arrowheads), which might be involved in cementing the larva to the substrate during settlement. (C) Later larval stage with setae pouch muscles (arrows), medioventral longitudinal muscles (empty arrows), and F-actin-rich area (arrowhead) on the dorsal side. (D) Metamorphic competent larva in ventral view with setae (se) and setae pouch muscles (arrows), which are ventrally interconnected by two strands of medioventral longitudinal muscles (empty arrows). The medioventral longitudinal muscles are interconnected by transversal muscles, which at this stage are concentrated into three bundles (asterisks). (E) Specimen during metamorphosis with remnants of larval setae pouches muscles (arrows) and larval medioventral longitudinal muscles (empty arrows), which are most probably undergoing resorption. The adult anterior adductor muscles (aad) start to develop. (F) Juvenile with mantle margin muscles (mm), anterior adductor muscle (aad), oblique muscle (ob), and posterior adductor muscles (pad). (G-I) Three-dimensional reconstruction of the dataset shown in D. (G) Ventral view of the musculature of a fully developed larva with medioventral longitudinal muscles (red), setae pouch muscles (yellow), and transversal muscle

(asterisk). (H) Same specimen as in G, anterior view. (I) Same animal as in G, dorsal view.

**Figure 2:** Semi-schematic representation of the larval musculature of craniiform and rhynchonelliform brachiopods. (A) Musculature of *Novocrania anomala* with setae pouch muscles (red circles), medioventral longitudinal muscles (white), and transversal muscles (yellow-grey). Size of the specimen is approximately 150  $\mu\text{m}$ . (B) Musculature of *Argyrotheca cordata* based on Altenburger and Wanninger (2009) with pedicle muscles (beige), longitudinal muscles (orange), central mantle muscles (brown), U-shaped muscle (green), setae pouch muscles (red circles), circular mantle muscle (light blue), serial mantle muscles (dark orange), setae muscles (purple), apical longitudinal muscles (dark blue), and apical transversal muscle (yellow). Size of the specimen is approximately 280  $\mu\text{m}$ .

**Figure 3:** Development of the serotonergic nervous system in *Novocrania anomala*. A, B, E, and F: Overlay of maximum projection micrographs of serotonin staining and light micrographs. C and D: Three-dimensional reconstruction of the dataset shown in B. Anterior faces upwards and scale bars equal 50  $\mu\text{m}$ . (A and B) Metamorphic competent larva with three pairs of setae bundles (se) and four flask-shaped serotonergic cells (asterisks) in the anterior part of the apical lobe (AL), as well as two ventral serotonergic neurites (arrows) running from the apical lobe towards the posterior lobe (PL). The stage in A is slightly younger than that depicted in B. C and D: Same dataset as in B with four flask-shaped serotonergic cells (red) and two ventral neurites which are interconnected

anteriorly (yellow). (C) Ventral view. (D) Lateral view. The flask-shape is visible only in one cell due to the different position of the cells. (E) Juvenile during metamorphosis with two ventral neurites (arrows) which run from the region of the former anterior lobe (AL) into the region of the former posterior lobe (PL). Larval setae (se) and juvenile shell (s) are present. (F) Later stage of a juvenile with two ventral neurites (arrows) which are interconnected by a commissure (co).

**Figure 4:** Development of the nervous system in *Novocrania anomala* as revealed by acetylated  $\alpha$ -tubulin staining. A–D: Overlay of maximum projection micrograph of  $\alpha$ -tubulin staining and light micrograph. E and F are 3D reconstructions of the dataset shown in D. Anterior faces upwards and scale bars equal 50  $\mu$ m. (A) First  $\alpha$ -tubulin signal in a juvenile five days after metamorphosis. The former larval apical lobe (AL) and posterior lobe (PL) are still visible under the shell (s) of the juvenile. Two ventral neurite bundles develop in the anterior lobe (arrows). The juvenile body is still covered by larval cilia (ci). Some serially arranged neurites (sn) extend inwards from the ventral neurite bundles. (B) The ventral neurite bundles (arrows) elongate further in posterior direction. A commissure (co) starts to form at the former border of the apical and the posterior lobe but is not yet closed. From the anterior portion of the ventral neurite bundles, serially arranged mantle neurites (smn) extend distally outwards and serially arranged neurites (sn) extend inwards. The cilia of the juvenile gut (gu) are visible in the median region of the juvenile. (C) Juvenile with the same structures as in B. The commissure (co) is closed and the ventral neurite bundles (arrows) have fused anteriorly. (D) Neural anatomy of a juvenile 17 days after metamorphosis with the commissure (co)

interconnecting the ventral neurite bundles (arrows) and the serially arranged mantle neurites (smn), which extend towards the edge of the juvenile mantle. (E) Three-dimensional reconstruction of the dataset shown in D, dorsal view. (F) Three-dimensional reconstruction of the dataset shown in D. Postero-dorsal view demonstrating that the ventral neurite bundles (yellow) and the serially arranged mantle neurites (green) bend ventrally.